Isolation and characterization of alpha amylase isolated from a hot water spring in Sri Lanka

Mathew C.D. and Rathnayake S.

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Colombo, Sri Lanka

ABSTRACT

This paper describes the purification and characterization of a thermostable α-amylase produced by G*eobacillus* sp. NMS 2 isolated from soil samples of a hot water spring in Nelumwewa, which is situated in the Polonnaruwa district of Sri Lanka. The microorganism isolated was identified by morphology, biochemical tests and 16S rRNA gene sequencing. Optimum extracellular enzyme production was 43 U/ml after 12 hours incubation at 50^{0}C. The enzyme was purified by extraction, ammonium sulphate fractionation and DEAE chromatography. The specific activity of the purified enzyme was 420 U/mg with a folds purification 2.8. Polyacrylamide gel electrophoresis showed only one protein band. From Lineweaver- Burke plot, the K_m and V_{max} values were 3.4 mg/ml and 460 μmolmin^{-1}mg^{-1} respectively. The enzyme was stable at temperatures from 10^{o}C to 60^{0}C with the optimum activity at 50^{0}C. *Geobacillus* sp. NMS 2 α-amylase showed optimum activity at pH 6.9 and it was stable at pH ranges from 6.9 to 9.

Keywords: Thermostable, α-amylase, Extracellular, G*eobacillus* sp. NMS 2

INTRODUCTION

Among the starch hydrolyzing enzymes α-amylase (EC 3.2.1.1) is a well known endoamylase. Endoamylases are able to cleave α,1-4 glycosidic bonds that are present in the inner part (endo) of the amylose or amylopectin chain. The end products of α-amylase action are an α-configuration linear and branched oligosaccharides with varying length. (Van der Maarel, et al., 2002). α- amylase constitute a class of industrial enzymes having approximately a 25% stake in the world enzyme market. Amylases are used in starch liquefaction process, paper, textile, bakery and detergent industries. (Gupta, et al., 2003) (Ahlawat, et al., 2009)

Amylases can be derived from several sources such as plants, animals and micro-organisms. However the enzymes from microbial sources are used in industries. Commercially α-amylase is mainly derived from the genus *Bacillus*. α-amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* are used in a number of industrial processes. (Pandey et al., 2000) These are thermophilic microorganisms and they secrete thermostable α-amylase. Thermostability is a desired characteristic of most industrial enzymes.

In the present study we report purification and characterization of thermostable α-amylase produced by thermophilic strain *Geobacillus* sp. NMS 2 which was isolated from soil samples of a hot water spring in Nelumwewa, which is situated in the Polonnaruwa district of Sri Lanka.

MATERIALS AND METHODS

Isolation of microorganism

Water and soil samples were collected from the hot water spring of Nelumwewa, in the Polonnaruwa district of Sri Lanka under sterile conditions. Samples were then inoculated into Starch-nutrient broth containing (g/l) Peptone, 5.0, Yeast extract, 2.0 Sodium chloride, 5.0, KCl, 0.5, $MgSO_4.7H_2O$, 0.5, $MnSO_4$, 0.04 $FeSO_4$,0.3, K_2HPO_4, 0.87, $CaCl_2$,$2.2x10^{-2}$ and soluble starch, 10.0,

pH 6.8 and incubated at 50^0C and 70^0C in an orbital shaker at 150 rpm overnight.

Identification of microorganism

Overnight culture were spread on starch- nutrient broth-agar medium containing agar(g/l) 15.0 pH 6.8. The bacterial species were identified by morphological, biochemical characterization according to Bergey's manual of determinative bacteriology (Holt, et al., 1994) and 16S rRNA gene sequencing. The morphological, biochemical characterization parameters included colony morphology with zone of clearance, gram properties, Kligler Iron medium reaction, Simmon's citrate assay, 6.5% NaCl assay, urease test, Methyl Red reaction, Voges Proskauer reaction, nitrate assay, indole assay, gelatin liquefaction, carbohydrate fermentation and hanging drop method for motility of the bacteria. Zone of clearance were observed flooding plates with 1% iodine in 2% KI. The clear zone around the colony indicated the amylase activity.

16S rRNA gene sequencing was done at "GENETECH" Institute in Sri Lanka. Total DNA was isolated from small colony of the sample and 5 µl of extracted DNA was subjected to the Polymerase chain reaction (PCR) using 27F/ 800R and 518F/1492R. Amplified DNA was subjected to DNA sequencing using 518F and 800R primers and the obtained DNA sequences were compared with already existing DNA sequences in NCBI Genebank.

Cultivation of isolated bacterial species at different temperatures

In order to select the best temperature for α-amylase production, bacterial species was cultivated in starch-nutrient broth at temperatures of 37^0C, 50^0C, 60^0C, and 70^0C for 48 hours and samples were collected at 4 hour intervals and determined optical density at 600 nm, α-amylase activity of supernatant, cell count and pH.

Purification of the enzyme

All purification steps were carried out at 4^0C.

Centrifugation of crude enzyme

After overnight incubation at 50^0C, bacterial broth medium was centrifuged at 10,000g for 15 minutes at 4^0C. Then measured the initial α-amylase activity, protein content and specific activity of supernatant.

Ammonium sulphate fractionation

Ammonium sulphate was added up to 30% saturation and centrifuge at 15,000g for 30 minutes. The supernatant obtained was brought up to 90% saturation and centrifuge at 15,000g for 30 minutes. The precipitate obtained was dissolved in a minimum volume of 0.02M phosphate buffer (pH 6.9) and dialyzed overnight against the same buffer. The dialyzed solution was centrifuged at 15,000g for 30 minutes and measured the volume, α-amylase activity, protein content and specific activity of the supernatant.

DEAE Sephadex ion exchange chromatography

The dialysed enzyme solution was applied to a DEAE Sephadex A-25 column, (2.5cm x 22.5cm) previously equilibrated with 0.02M phosphate buffer (pH 6.9) and the column was eluted with a 1:1 gradient 0.04M to 0.2M NaCl in 0.02M phosphate buffer (pH 6.9).The flow rate was 60ml per hour. Fractions (10ml) were Collected and tested for α-amylase activity and absorbance was measured at 280 nm. The fractions having high α-amylase activity were pooled.

Alpha amylase assay

α-amylase activity was assayed by the method described by Bernfeld (1955). One unit of α-amylase is defined as the amount of enzyme which releases one µmol of reducing sugar, per minute with glucose as the standard.

Protein estimation

Protein content was determined by Lowry's precipitation method (Lowry et al., 1951) using Bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis

The purity of alpha amylase eluted from DEAE ion exchange chromatography was determined by polyacrylamide gel electrophoresis by the method described by Davis (1964).

Characterization of alpha amylase

All the assays were done in triplicates.

Effect of temperature on the alpha amylase activity

To study the effect of temperature on enzyme activity, 1% (w/v) starch solution and purified α-amylase were incubated for 15 minutes over the range of 10^0C to 80^0C. Assayed the activity α-amylase at respective temperature.

Stability of the enzyme at different temperature

Enzyme solutions were incubated temperature for 10^0C to 80^0C for one hour. Then these enzyme solutions were kept them at 50^0C for 20 minutes. Assayed the α-amylase activity at 50^0C.

Effect of pH on the alpha amylase activity

The optimum pH of the enzyme was determined by varying the pH of assay reaction from pH 3 to 10 at 50^0C using, 0.02M citrate/ phosphate buffer (pH 3, 4 and 5), 0.02M sodium phosphate buffer (pH 6, 6.9 and 8) and 0.02M carbonate/ bicarbonate buffer (pH 9 and 10).

Stability of the enzyme at different pH

To measuring the pH stability, the enzyme was incubated in different buffer solutions (pH 3-10) at 50^0C in the water bath for one hour. Assayed α-amylase activity at pH 6.9.

Kinetic analysis

Kinetic constants were detected by varying the starch concentration from 2 $x10^{-3}$ to 1 $x10^{-2}$ g/ml and assaying α-amylase activity. V_{max} and K_m values were calculated using the Lineweaver- Burke plot.

RESULTS

Isolation of the microorganisms

Both water and soil samples from a hot water spring, showed high optical density at 50^0C but at 70^0C only the soil sample showed high optical density at 600 nm. Therefore the bacterial culture obtained from soil sample was used for further studies. Soil microorganisms were spread on starch-nutrient agar plates and incubated at 50^0C overnight. The morphology indicated the presence of a single type of bacterial colony.

Morphological and biochemical characterization of isolated bacterial species

Isolated bacterial colony was yellow colored, medium sized, rounded, convex, smooth, opaque and mucoid colonies. The colonies showed a clear zone of 0.5 mm diameter with iodine test (Figure 1). Morphological studies showed a gram positive, rod shaped cells with ability to form cylindrical endospores (Figure 2). Results of the biochemical tests are given in Table 1.

Identification by 16S rRNA gene sequencing

16S rRNA gene sequencing (carried out by GENETECH, Sri Lanka) indicated the results are highly compatible (98%) with genus *Geobacillus* (Figure 3).
According to 16S rRNA gene sequencing and biochemical characterization bacteria was identified as *Geobacillus* sp. NMS 2.

Effect of temperature on α-amylase production

Geobacillus sp. NMS 2 was cultivated at 37^0C, 50^0C, 60^0C, and 70^0C and maximum α-amylase production was observed at 50^0C (Figure 4). Increasing and decreasing the temperature from optimum temperature resulted in decreasing the production of enzyme. At 50^0C maximum α-amylase activity of the supernatant was 43 U/ml at 12 hours of incubation and pH of the culture medium decreased from 6.9 to 4.6 after 32 hours incubation (Figure 5).

Enzyme purification

The results obtained from enzyme purification are given in Table 2. DEAE Sephadex gel chromatography showed a single enzyme peak (Figure 6). The pooled enzyme activity peak had a specific enzyme activity of 420 U/mg with a purification fold 2.8 and recovery of 70%. Polyacrylamide tube gel electrophoresis showed one protein band indicating that the enzyme was homogeneous (Figure 7).

Characterization of purified alpha amylase

Effect of temperature on the alpha amylase activity

The effect of temperature on the *Geobacillus* sp. NMS 2 α-amylase showed a optimum activity at 50^0C. The relative activities at 40^0C and 60^0C respectively were approximately 80% and 90.5% of the activity at 50^0C (Figure 8).

Stability of the enzyme at different temperature

Incubation of the enzyme for one hour at different temperatures showed that enzyme was stable at temperatures ranging from 10^0C to 60^0C. But at 70^0C 34% of its maximum activity was lost and at 80^0C 41% of its maximum activity was lost (Figure 9).

Effect of pH on the alpha amylase activity

Enzyme activity was determined at pH values from 3 to 10. The optimum pH was at 6.9 and high activity was observed from 6 to 10 (Figure 10).

Stability of the enzyme at different pH

The α-amylase was stable over the pH range of 6.9 to 9. After incubation of the enzyme for one hour at pH 3 to 10 the original activity at pH 6.9 decreased by 30% at pH 3 and at pH 10 the decrease was 28% (Figure 11).

Determination of kinetic values

From the Lineweaver- Burke plot, the K_m and V_{max} values were 3.4 mg/ml and 460 µmol/min/mg of protein at 50^0C and pH 6.9 (Figure 12).

Figure 1. Formation of clear zone around the bacterial colony with iodine test.
Bacteria were grown on a starch-nutrient agar medium and incubated at 50^0C for overnight.

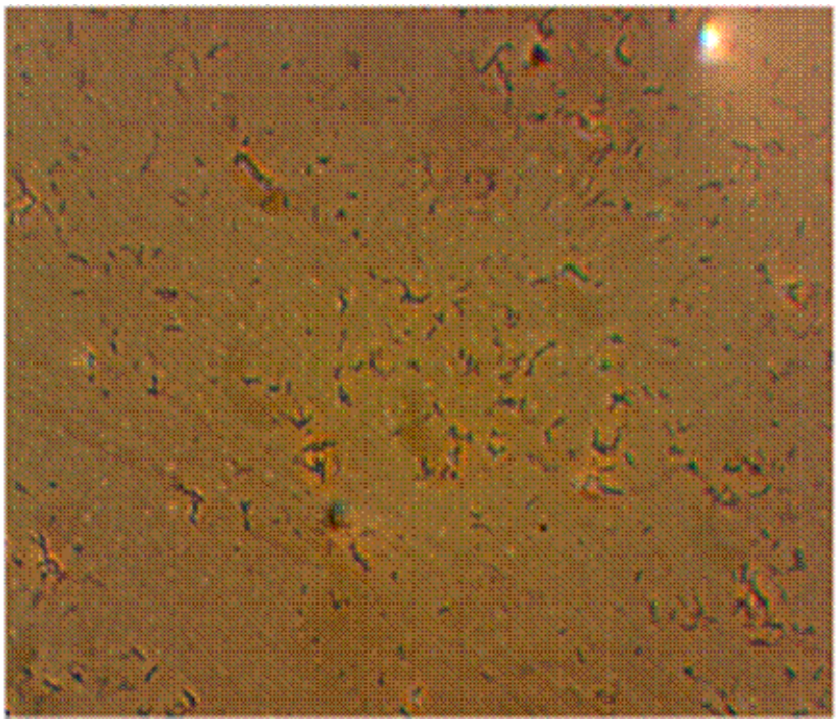
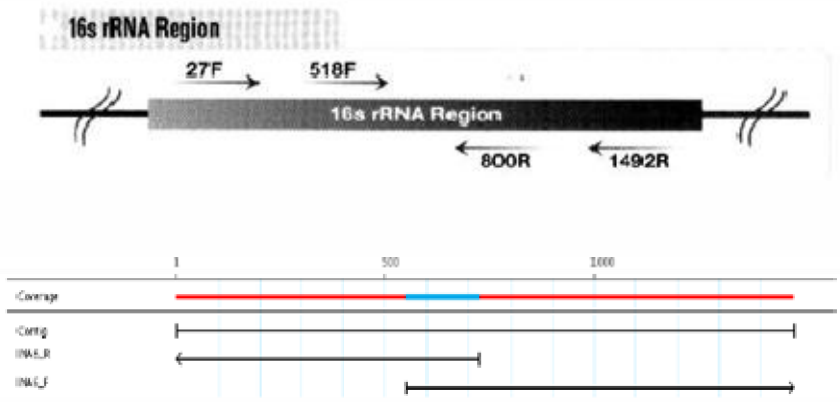


Figure 2. Gram stain of bacteria view under inverted fluorescence microscope (Olympus, 1x70-S1F2) (magnification x400)

Table 1. Gram stain and biochemical tests results for bacterial species

Gram properties Gram stain	Gram positive rod shaped bacilli with one cylindrical endospore per cell
Size	Length varied from 8 to 10 µm with 1µm diameter
Motility Test	Highly motile
Biochemical tests KI medium	Alkaline slant and Acid butt (K/A) with no gas
Simmon's citrate	Positive
6.5% NaCl	Positive
Urease Test	Negative
Methyl red	Negative
Voges Proskauer	Negative
Nitrate	Positive
Indole	Negative
Gelatin liquefaction	Negative
Carbohydrate fermentation Glucose	Positive, no gas
Sucrose	Positive, no gas
Maltose	Positive, no gas
Mannitol	Positive, no gas
Lactose	Negative

According to Bergey's manual of determinative bacteriology, morphology and biochemical characterization showed isolated bacterial sp. belonged to genus *Geobacillus* sp.



Result : Sequence of sample NMS 2

5'CTTAAGTCTGATGTGAAAGCCCACGGCTTAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAGAGCG
GAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGC
TGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGG
GGTTATCCCTTTAGTGCTGTAGCTAACGCGTTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCTGACA
ACCCTGGAGACAGGGCGTTCCTCCCTTCGGGAGGACAGGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCCTAGTTGCCAGCATTCAGTTGGGCACTCTAGGGGGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCGGTACAAA
GGGCTGCGAACCCGCGAGGGGGAGCGAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGC
CGGAATCGCTAGTAATCGCGGATCACATGCCGGGGGGAAAAAACCTTTCCCGGGGCTTTGTACACACCGCCCGTCACACCACG
AGAGCTTGCAACACCCGAAGTCGGTGAGGTAACCCGCAAGGGAGCCAGCCGCCGAAGGTGGGGCAAGTGATTGGGTGA3'

Figure 3. 16s rRNA gene sequence of *Geobacillus* sp. NMS 2

27F/ 800R and 518F/1492R primers used to the Polymerase chain reaction (PCR) and amplified DNA was subjected to DNA sequencing using 518F and 800R primers. Arrows indicate the primer's directions.

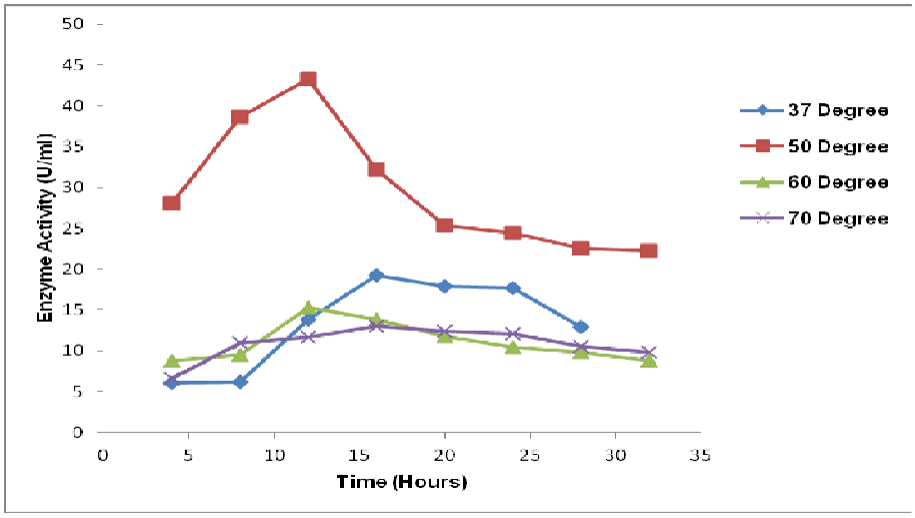


Figure 4. Effect of temperature on α-amylase production

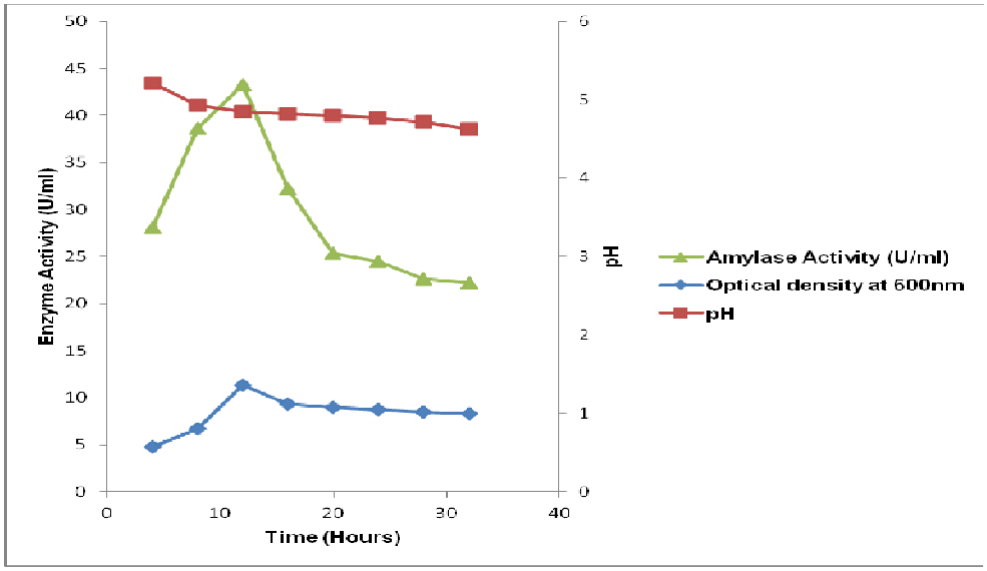


Figure 5. Bacterial growth (Optical density -600 nm), α-amylase activity and pH of *Geobacillus* sp. NMS 2 at 50^{0}C

Table 2. Summary of the purification of alpha amylase from *Geobacillus* sp. NMS 2

Purification step	Enzyme Activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Recovery (%)	Purification Fold
Culture Supernatant	41.0	0.27	152	100	1
90% $(NH_4)_2SO_4$ saturated pellet	34	0.105	324	83	2
DEAE A-25 ion exchange chromatography	28.6	0.068	420	70	2.8

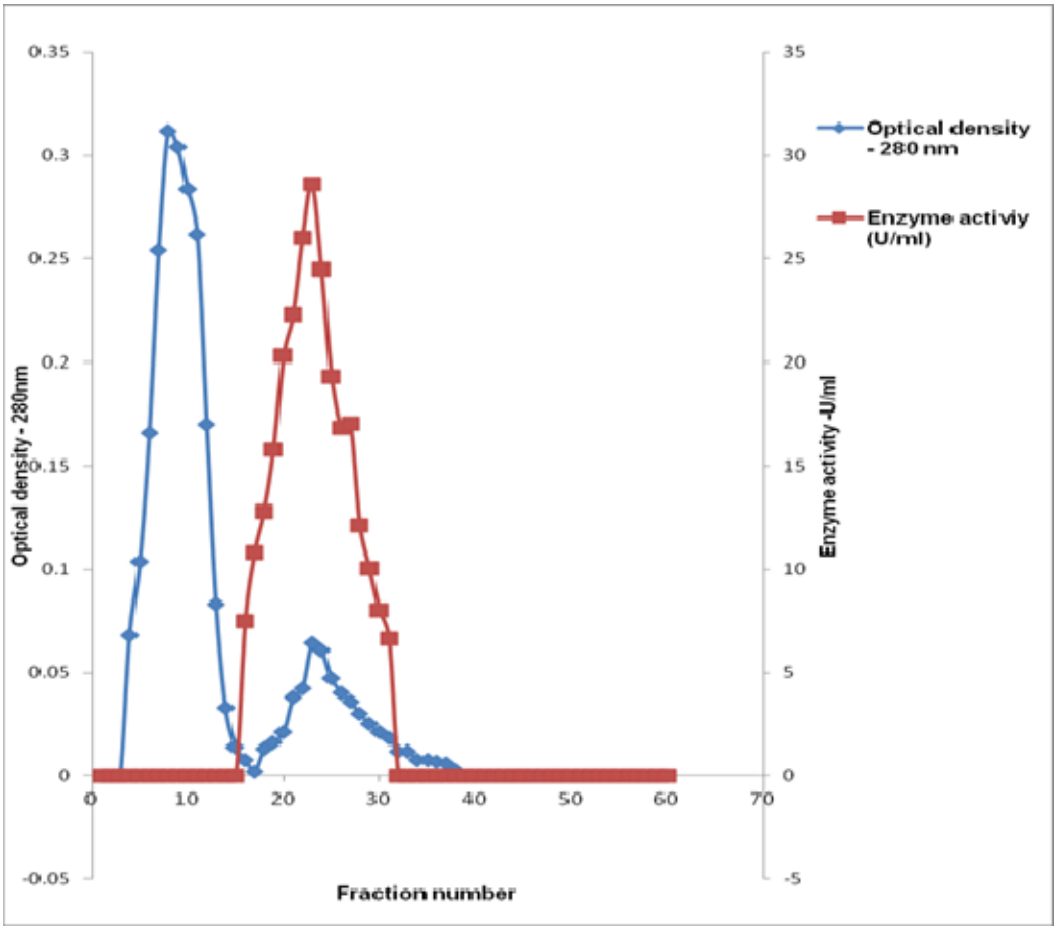


Figure 6. Fraction eluted by ion-exchange chromatography showing one peak of enzyme activity

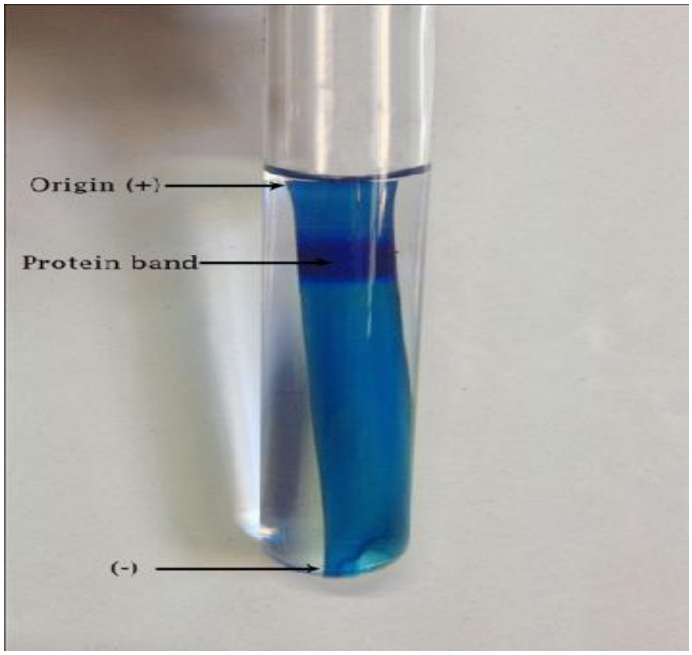


Figure 7. Polyacrylamide gel electrophoresis of *Geobacillus* sp. NMS 2 α- amylase

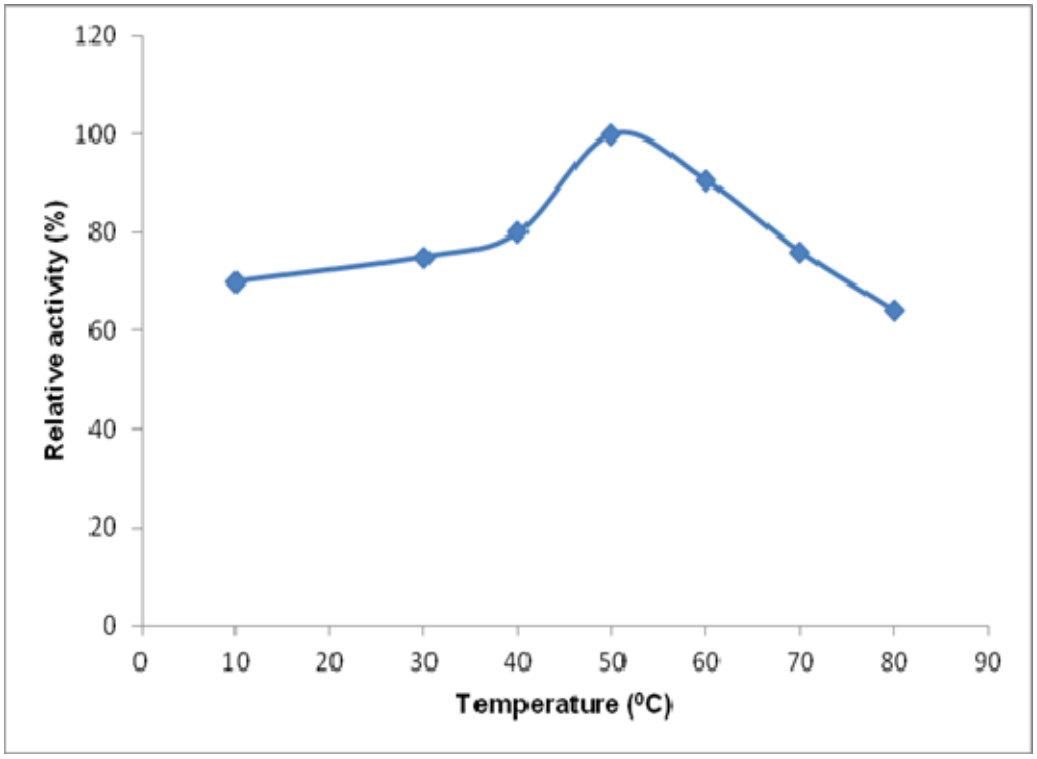


Figure 8. Effect of temperature on the activity of purified alpha amylase from *Geobacillus* sp. NMS 2

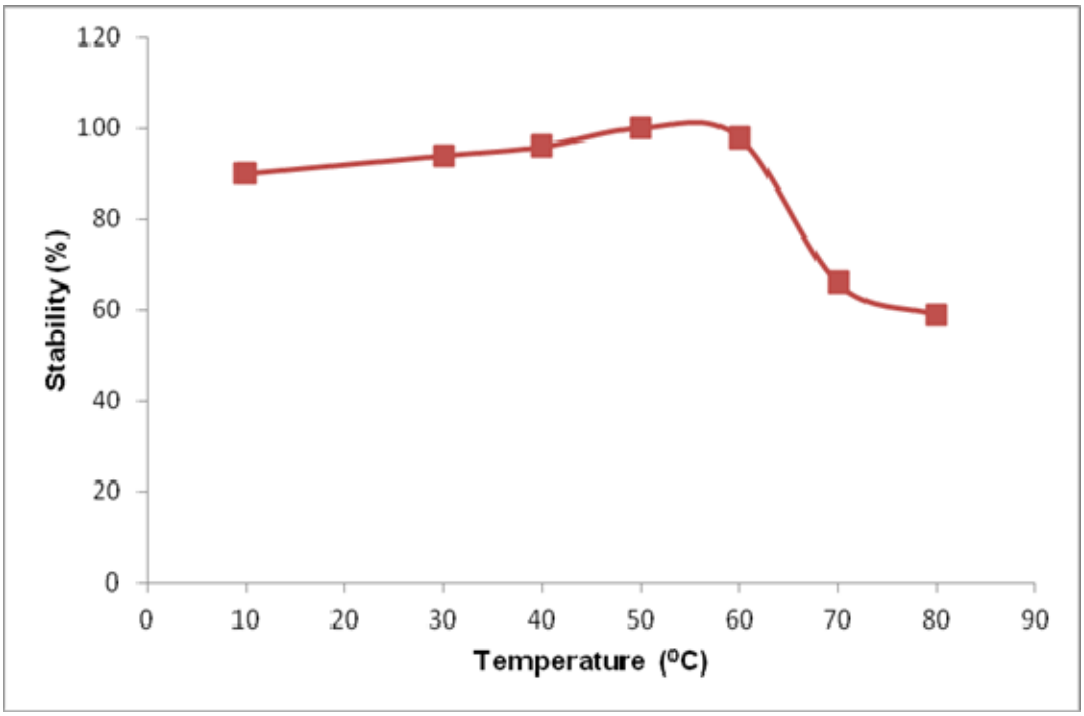


Figure 9. Temperature stability of purified alpha amylase from *Geobacillus* sp. NMS 2

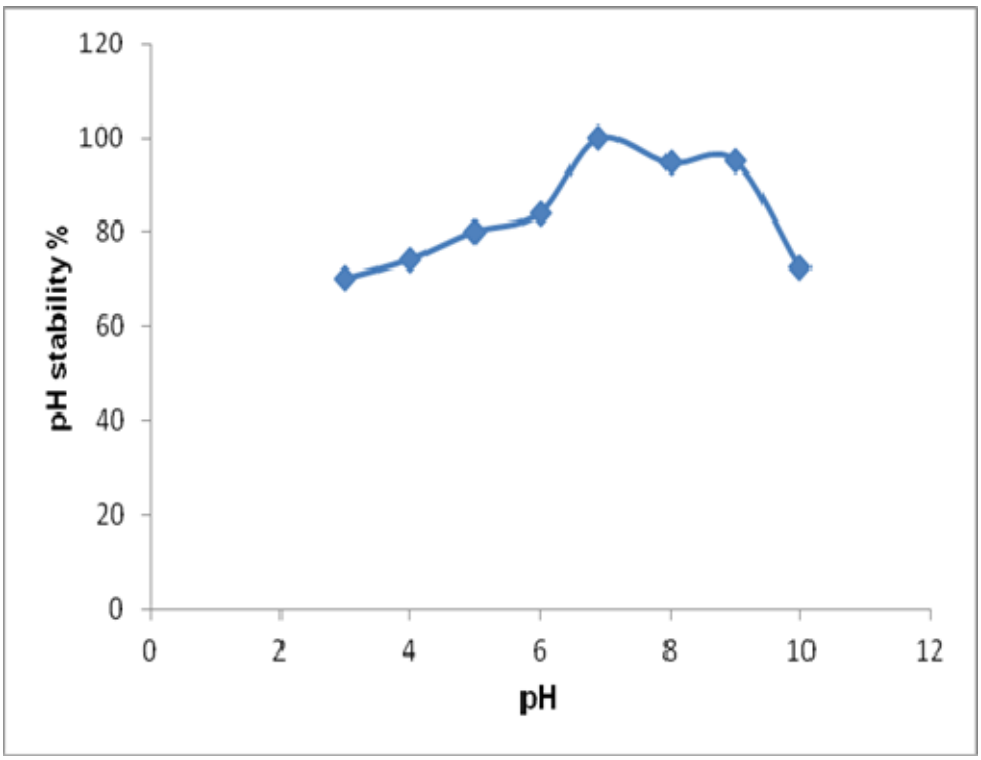


Figure 10. Effect of different pH values on the activity of purified alpha amylase from *Geobacillus* sp. NMS 2

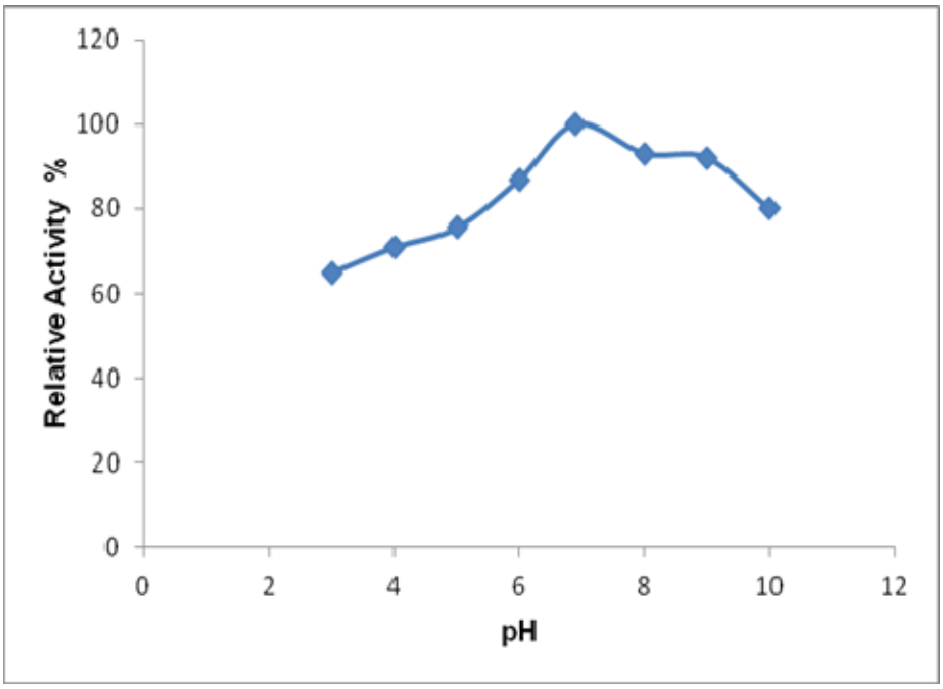


Figure 11. pH stability of the purified alpha amylase from *Geobacillus* sp. NMS 2

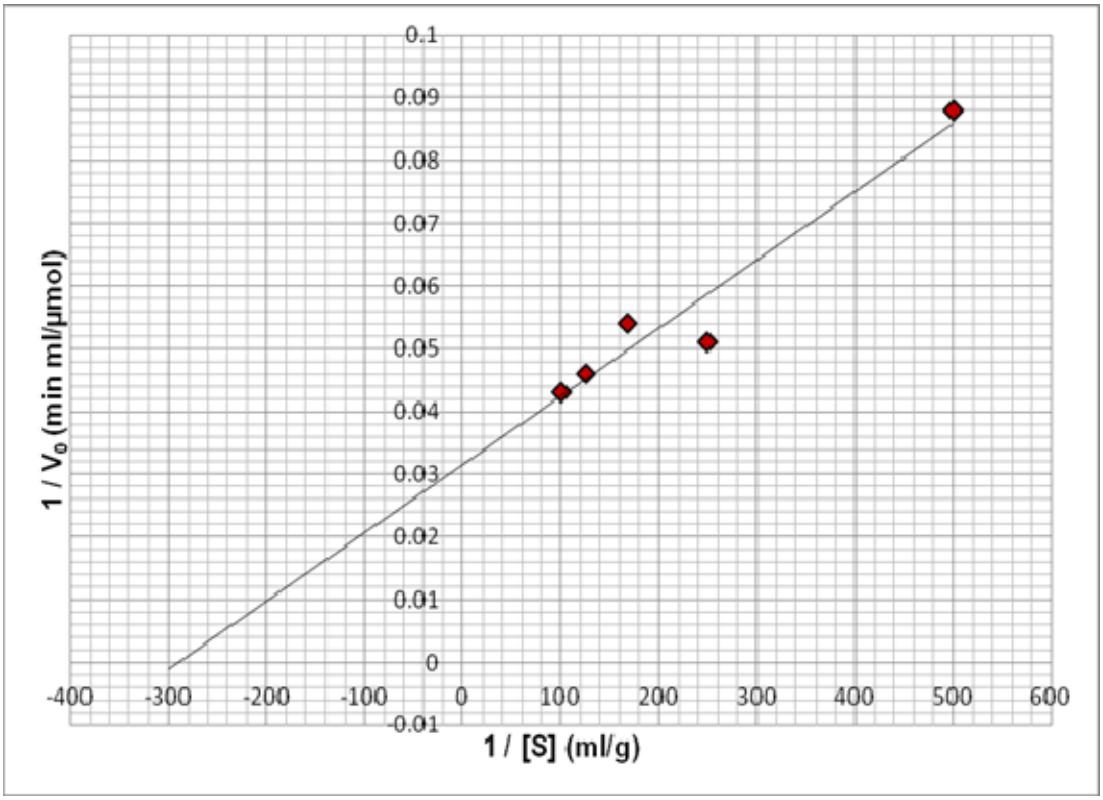


Figure 12. Lineweaver- Burke plot of reciprocal of starch concentration versus initial velocities used for determination of K_m and V_{max} values of alpha amylase from *Geobacillus* sp. NMS 2

DISCUSSION

Bacillus species such as *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis* and *Bacillus amyloliquefaciens* are the most important sources of α-amylase. (Pandey et al., 2000) In current study we isolated thermophilic microorganisms from both water and soil sample of the hot water spring at 50^{0}C and also at 70^{0}C. But at 70^{0}C low growth was observed from water samples. These microorganisms are facultative thermophiles which can grow only between 37^{0}C to 60^{0}C. (Kikani et al., 2010) Microorganisms from soil exhibited growth at 70^{0}C. (Kikani et al., 2010) Because of its high temperature tolerance we isolated the microorganisms from soil samples.

Geobacillus sp. NMS 2 was showed clear zone of 0.5 mm diameter with iodine test on starch-nutrient agar plate. But a newly isolated *Geobacillus* sp. IIPTN from the hot spring water of Uttarakhand's Himalayan region in India, showed clear zone of more than 1.0 cm diameter with iodine test. (Dheeran et al., 2010) *Geobacillus* sp. IIPTN showed high α-amylase activity about 192 U/ml, was much higher than our present study. *Bacillus amyloliquefaciens* and *Bacillus subtilis* had clear zone of 39.5mm and 29.5 mm respectively, while the bacteria isolated from soil showed much lesser (5.5 mm) zone of clearance (Khan et al., 2011).

Temperature is a highly sensitive parameter for α-amylase productivity. The influence of temperature on amylase production is related to the growth of the microorganism.(Gupta, et al., 2003) In this study the production of α-amylase was determination by growing the bacteria at different temperatures ranging from 37^{0}C to 70^{0}C. The optimum enzyme productivity (43 U/ml) was observed at 12 hours of cultivation at 50^{0}C. Similarly Riaz and coworkers reported 50^{0}C was the optimum temperature for production of α-amylase from the *Bacillus subtilis* KIBGE-HAR (Riaz et al., 2009) and also maximum production of α-amylase (15.27 U/ml) was obtained at 50^{0}C from the *Geobacillus*(*Bacillus) stearothermophilus* NCIM 2922. (Talekar and Patil, 2012) But some of the thermophiles produced α-amylase which showed optimum temperature above 50^{0}C. (Carvalho et al., 2008), (Dheeran et al., 2010)

According to our results, cellular growth of *Geobacillus* sp. NMS 2 (optical density at 600 nm) and α-amylase production, were affected by the time of incubation. It was observed that maximum α-amylase production occurred when the bacterial growth reached the stationary phase. (Vieille et al., 1996) Other investigators also have reported similar results (Bajpai, 1989), (Saito and Yamamoto, 1975).

Amylase production by many *Bacillus* sp. was known to be affected by the amount of dissolved oxygen in the

medium. (Riaz et al., 2009) After the optimum temperature of at 50^{0}C, α-amylase production by *Geobacillus* sp. NMS 2 decreased at higher temperatures. This might be due to an elevated temperatures, the solubility of oxygen was decreased. (Campbell and Pace, 1968) This limitation of dissolved oxygen affected the production of α-amylase, because it was reported that different levels of oxygen in the medium induced changes in the surface protein layer (S-layer) of bacterial cell membrane. (Sara et al., 1996) This is involved in the control of the release of enzyme as extracellular enzyme.

Initial pH (6.9) of the medium was changed with the growth of the bacteria. At 50^{0}C it decreased to pH 4.6 after 32 hours of incubation.

In current study 90% ammonium sulphate saturation used for precipitation of α-amylase from *Geobacillus* sp. NMS 2. Considering to the finding of other workers, α-amylase from mutant strain of *Bacillus* species was partially purified by 95% ammonium sulphate saturation.(Ashraf, 2004), 80% ammonium sulphate saturation was used for α-amylase from *Bacillus cereus* M.K (Mrudula and Kokila, 2010) and *Bacillus* sp. Ferdowsicous (Asoodeh et al., 2010). α-amylase of *Geobacillus*(*Bacillus) stearothermophilus* NCIM 2922 was partially purified by 70% ammonium sulphate saturation. (Talekar and Patil, 2012)

Fractions collected by DEAE - Sephadex ion exchange chromatography showed a specific activity of 420 U/mg of protein. α-amylase from *Bacillus cereus* strain, purified using DEAE Sepharose column and FPLC chromatogram had a specific activity of 50 U/mg of protein. (Mahdavi, et al., 2010) *Bacillus* sp. Ferdowsicous α-amylase showed a specific activity 267 U/mg of protein after purification on Q- Sepharose column chromatography. (Asoodeh et al., 2010). After purification of α-amylase from *Bacillus licheniformis* EMS-6 by 80% ammonium sulphate saturation and FPLC chromatography using RESOURSE-S column, showed specific activity as 2,665 U/mg of protein. (Ul-Haq et al., 2010)

Regarding the factors affecting the *Geobacillus* sp. NMS 2 α-amylase, the optimum temperature for the α-amylase activity was 50^{0}C. Other investigators have reported temperatures optima between in 30 to 80^{0}C. Mahdavi and coworkers showed *Bacillus cereus* strain α-amylase was stable up to 70^{0}C while the optimum temperature was 50^{0}C. But rapid loss of activity occurred above 70^{0}C. (Mahdavi et al., 2010) *Bacillus subtilis* BS5 α-amylase retained 30% of its activity when heated for 10 minutes at 80^{0}C and it exhibited maximum activity at 50^{0}C. (Femi-ola and Olowe, 2010) *Geobacillus* sp. IIPTN produced hyperthermostable α-amylase which was stable between 40 to 120 ^{0}C with an optimum activity at 80 ^{0}C. (Dheeran et al., 2010)

Geobacillus sp. NMS 2 α-amylase was stable for a pH range of 6.9 to 9. Maximum activity was observed at pH 6.9. Most of the *Bacillus* sp. α-amylase has shown maximum activity in the pH ranges of 6.0 to 7.0 or 5.0 to 7.0 (Gupta, et. al., 2003) Partially purified α-amylase from *Geobacillus*(*Bacillus) stearothermophilus* NCIM 2922 have a optimum pH of 6.5. (Talekar and Patil, 2012) However α-amylase from thermophilic *Bacillus* species strain SMIA-2 has optimum activity at pH 8.5. (Carvalho, et. al., 2008)

In this study, the K_m and V_{max} values were found to be 3.4 mg/ml and 460 µmol/min/mg, respectively from the Lineweaver- Burke plot. High K_m values were reported for α-amylase from *Lactobacillus manihotivorans* as 3.44 mg/ml and V_{max} value was 0.45 mg hydrolyzed starch/ml/min. (Goyal et al., 2005) Ezeji and Bahl (2006) reported for purified α-amylase from *Geobacillus thermodenitrificans* HRO 10, K_m and V_{max} values of 3.05 mg/ml and 7.35 U/ml respectively. For *Geobacillus*(*Bacillus) stearothermophilus* NCIM 2922 α-amylase K_m and V_{max} values were 1.7 mg/ml and 112.8 µmol/min respectively (Talekar and Patil, 2012).

CONCLUSION

α-amylase from *Geobacillus* sp. NMS 2 indicated the enzyme was thermostable from 10 to 60^{0}C and showed optimum activity at 50^{0}C and pH 6.9. Thermostability is one of the important characteristics for industrial applications. Thus this α-amylase can be used for industrial purpose. The pH stability between 6.9 to 9 makes this suitable for detergent industry. In this research we studied temperature involvement for production of α-amylase. Also the study of effect of initial pH, type of carbon source and effect of metal ions can be used to enhance enzyme production.

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